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Coexistence of Copy Number Increases of *c-Myc*, *ZNF217*, *CCND1*, *ErbB1* and *ErbB2* in Ovarian Cancers

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Key Words
Ovarian cancer · Oncogenes · Genomic imbalances · Fluorescence in situ hybridization

Summary
Background: We selected 5 oncogenes with well-established roles in carcinogenesis – *CCND1*, *ErbB1*, *ErbB2*, c-*myc* and *ZNF217* – to investigate the coexistence of their copy imbalances in relation to the clinico-pathological characteristics of ovarian tumors. **Materials and Methods:** Fluorescence in situ hybridization for the 5 genes was applied to a preexisting tissue microarray. 38 ovarian tumors were successfully analyzed for copy number changes of the 5 genes. **Results:** At least one of these oncogenes was gained/amplified in 27 out of 38 tumors (71.1%). We report the highest frequency of *c-myc* genetic gain/amplification since it affected 42.1% of the ovarian tumors. We observed sequential involvement of copy number alterations of the other genes in the presence of *c-myc* disruption. The incidence of copy number changes of the 5 oncogenes – both single and combinatorial – was higher in high-grade tumors. All double aberrations in the serous group comprised *c-myc* and *ZNF217* copy number increases. **Conclusions:** Our results revealed a combination between copy number increases of *c-myc* and *ZNF217*, associated with serous histology. The data from this combined analysis of the 5 oncogenes could be used as a basis in considering the combined approach in molecular-based therapy of ovarian cancer.

Schlüsselwörter
Ovarialkarzinom · Onkogene · Ungleichgewicht, genomisches · Fluoreszenz-in-situ-Hybridisierung

Zusammenfassung
Hintergrund: Wir haben 5 Onkogene, deren Rolle in der Karzinogenese etabliert ist (*CCND1*, *ErbB1*, *ErbB2*, *c-myc* und *ZNF217*), ausgewählt, um die Koexistenz von Kopiezahl-Ungleichgewichten in Bezug auf klinisch-pathologische Eigenschaften von Ovarialtumoren zu untersuchen. **Material und Methoden:** Zur Detektion dieser 5 Gene wurde die Fluoreszenz-in-situ-Hybridisierung auf ein schon existierendes Gewebe-Microarray angewandt. 38 Ovarialtumoren wurden erfolgreich auf Veränderungen der Kopienzahl dieser 5 Gene untersucht. **Ergebnisse:** Bei 27 von 38 Tumoren (71,1%) war mindestens eines dieser Onkogene vermehrt/amplifiziert. Die höchste Frequenz der genetischen Vermehrung/Amplifikation wurde für *c-myc* gefunden, da dies für 42,1% der Ovarialtumore zutraf. Im Falle einer *c-myc*-Mutation konnten wir eine sequenzielle Involvierung einer Veränderung der Kopienzahl der anderen Gene beobachten. Das Auftreten veränderter Kopienzahlen der 5 Onkogene – einzeln oder in Kombination – war bei Tumoren höheren Grades verstärkt. Alle Doppelaberrationen in der Gruppe der serösen Tumoren umfassten Erhöhungen in der Kopienzahl von c-*myc* und *ZNF217*. **Schlussfolgerungen:** Unsere Ergebnisse zeigen eine Verknüpfung der Kopienzahlerhöhungen von *c-myc* und *ZNF217* sowie eine Assoziation mit seröser Histologie. Die Daten aus dieser kombinierten Analyse von 5 Onkogenen könnten als Basis für die Entwicklung einer kombinierten molekularen Therapie bei Ovarialkarzinomen verwendet werden.

Introduction

Ovarian carcinoma has the highest mortality of all gynecological cancers. Close to 75% of the patients have advanced-stage disease at diagnosis [1]. Their prognosis is correlated with numerous clinical and biological factors. The sequential transformation of normal epithelium to invasive carcinoma is reflected by increasing levels of cellular alterations.

Most ovarian carcinomas show complex chromosome aberrations with hypodiploid or near-triploid stem-line copy number [2]. The average number of copy alterations index is a measure for the number of acquired chromosomal copy alterations in a given histological type or at specific stages [3]. The accumulated data from ovarian carcinoma suggested the presence of at least three modes of karyotypic evolution in this tumor type: phase I with 1–7 imbalances (corresponding to low stage and grade), phase II with 8–15 imbalances (typical of moderate stage and grade), and phase III with > 15 imbalances (corresponding to high stage and grade) [4]. Recently, high-resolution analysis by whole genome arrays revealed > 380 small regions of copy number gain or loss (< 500 kb) among 31 primary ovarian cancers; the average number of small-region copy imbalances was 12 [5]. Array comparative genomic hybridization (CGH) analysis of 26 ovarian cancers with 1300 genomic clones identified twice as many imbalances in the group of ovarian tumors resistant to standard chemotherapy (55 losses/gains) compared to the sensitive group (28 losses/gains) [6]. In general, the number of chromosomal aberrations clearly correlates with disease behavior and progression and seems to be a valid marker for increased genomic instability.

It would be exceedingly interesting to analyze if given specific copy number alterations occur as a sequence and reflect a pattern of stage-, grade- or histology-specific parameters.

We selected five oncogenes – *CCND1, ErbB1 (EGFR), ErbB2 (HER2), c-myc* and *ZNF217* – for combinatorial fluorescence in situ hybridization (FISH) analysis of their copy number changes in a subset of 38 ovarian tumors with different histologies, stages and grades. All of them have a well-established role in carcinogenesis. *CCND1* is a growth-regulatory gene; in the G1 phase of the cell cycle, *CCND1* together with its cyclin-dependent kinase (cdk) partner is responsible for the transition to the S phase by phosphorylating the product of the retinoblastoma gene (pRb), which then releases transcription factors [7]. The HER family members EGFR and ErbB2 are receptors of growth factors with an important role in normal cell growth and differentiation [8]. c-Myc is a transcription activator of proliferation factors and it is also an inhibitor of the factors, suppressing growth [9]. *ZNF217* encodes a Kruppel-like zinc-finger transcription factor and was originally identified based on its core location in the 20q13.2 amplicon observed in some breast and ovarian cancers [10].

The aim of this study was to investigate the coexistence of copy imbalances of these oncogenes in relation to the clinicopathological characteristics of ovarian tumors.

Materials and Methods

Ovarian Tumor Tissue Microarray

A preexisting tissue microarray (TMA) containing 235 archived ovarian tumor samples (from formalin-fixed paraffin-embedded tissue blocks) from 235 patients, collected during the period of 1999–2004, was used. The ovarian tumors varied considerably in their histological type. The TMA contained specimens from all histological variants: serous, mucinous, endometrioid, clear cell tumors, Brenner tumors, mixed epithelial tumors, undifferentiated, unclassifiable, granulose cell tumors, germline cell tumors (teratomas, dysgerminomas). The slides of all tumors were reviewed prior to construction by a single pathologist. Tumor stage and grade were defined according to Federation of Gynecology and Obstetrics (FIGO) and World Health Organization (WHO) criteria [11].

For TMA construction, a hematoxylin and eosin (H&E)-stained section was made from each block to define representative tumor areas. Tissue cylinders with a diameter of 0.6 mm were punched from tumor areas and brought into a recipient paraffin block using a custom-made precision instrument [12, 13]. Samples were distributed into two regular-sized recipient paraffin blocks, each containing about 500 specimens. 5-µm sections of the blocks were transferred to glass slides using a paraffin-sectioning aid system (adhesive coated slides, adhesive tape, UV lamp; Instrumedics Inc., Hackensack, NJ, USA).

FISH

Prior to hybridization the slides were treated with the Paraffin Pretreatment Reagent Kit (Vysis). FISH was performed using the following probes: HER-2 (erbB2) Spectrum Orange/CEP17 Spectrum Green-labeled probe (Cat #30–161060, Vysis), ERBB1 Spectrum Orange/CEP7 Spectrum Green-labeled probe (Cat #32–191053, Vysis), Cyclin D1 Spectrum Orange/CEP11 Spectrum Green-labeled probe (Cat #32–191039, Vysis), c-myc Spectrum Orange (Vysis, Cat #30–190006), ZNF217 Spectrum Orange (Vysis, Cat #32–190016). Denaturation of the DNA was carried out at 75 °C for 10 min (probe mixture) or 5 min (slides). The probe mixture was applied to the slides and hybridized overnight in a moist chamber at 37 °C. The posthybridization washes were performed as described in the 'LSI procedure' (Vysis). Slides were counterstained with DAPI in antifade. The presence of > 6 signals (or 3 times more gene signals than centromere signals per cell) or tight clusters in at least 10% of tumor cells was considered as amplification according to the instruction of Vysis (Pathvysion). Presence of > 2 signals (or more gene signals than centromere signals) in at least 10% of tumor cells not reaching the threshold (3 ×) was considered a 'gain'. Genetic gain refers to chromosomal aberrations in the cell (numerical or structural): aneusomies, translocations, duplications, etc.

Statistics

Contingency table analysis and χ^2 tests were used to study the relationship between gene copy number changes and tumor phenotype, i.e. histology, tumor grade and stage.

Results

Incidence of Copy Number Changes of the Analyzed Genes

Totally, 38 ovarian cancers were successfully analyzed for copy number changes of *CCND1*, *ErbB1*, *ErbB2*, *c-myc* and *ZNF217*. At least one of these oncogenes was gained/amplified in 27 out of 38 tumors (71.1%). The copy number changes of these oncogenes varied from 0 (in 28.9% of tumors), 1 (in 39.5%), 2 (in 18.4%), 3 (7.9%) to 4 (in 5.3% of tumors) per sample. The type of aberrations, combinations and pathological characteristics of the tumors are represented in table 1.

Table 1. Clinico-pathological data and copy number changes in ovarian tumors with aberrations.

Histology	Grade	Stage	Aberration (G = gain/A = amplification)
Serous	low	III	ZNF217 (G)
Serous	low	I	c-myc (G) + ZNF217 (G)
Serous	low	III	c-myc (G) + ZNF217 (G)
Serous	low	I	c-myc (G) + ZNF217 (G) + ErbB2 (G)
Serous	low	I	c-myc (G) + ZNF217 (G) + ERBB1 (G) + CCND1 (A)
Serous	high	III	ZNF217 (G)
Serous	high	I	c-myc (G)
Serous	high	III	c-myc (G)
Serous	high	I	c-myc (A)
Serous	high	I	c-myc (G) + ZNF217 (G)
Serous	high	III	c-myc (G) + ZNF217 (G)
Serous	high	III	c-myc (G) + ZNF217 (G)
Serous	high	II	CCND1 (G) + ErbB2 (G)
Serous	high	III	c-myc (G) + ZNF217 (G) + ErbB2 (G)
Mixed	low	II	ZNF217 (G)
Mixed	low	II	c-myc (A)
Mixed	low	III	c-myc (A)
Endom.	low	II	CCND1 (G)
Clear cell	low	III	c-myc (G) + ZNF217 (G) + ErbB2 (A) + ERBB1 (G)
Mixed	high	I	ERBB1 (A)
Undiff.	high	III	CCND1 (G)
Undiff.	high	I	ZNF217 (G)
Undiff.	high	III	ZNF217 (G)
Undiff.	high	III	c-myc (G)
Endom.	high	I	ERBB1 (G)
Dysgerm.	high	III	ERBB1 (G) + ErbB2 (G)
Undiff.	high	III	c-myc (G) + ZNF217 (G) + ErbB2 (G)

Fig. 1. Sequential involvement of copy number changes of the analyzed genes in ovarian tumors: The baseline indicates all ovarian samples; the next lines represent respectively single, double, triple and 4 aberrations involving *c-myc*. *c-Myc* alterations are present here in all aberrant tumors; *ZNF217* alterations occurred in all combined aberrations with *c-myc*.

Single and Combinatorial Gene Alterations

We established copy number increases (both single and combined) of *c-myc* in 42.1% of ovarian cancers, of *ZNF217* in 39.5%, of *ErbB1* in 13.2%, of *ErbB2* in 13.2%, and of *CCND1* in 10.5% of the tumors. The most frequent single aberration was a gain/amplification of *c-myc*. It occurred in 6 out of 38 ovarian tumors (15.8%), followed by *ZNF217* (in 13.2%), *ErbB1* and *CCND1* (both in 5.3% of the tumors). None of the ovarian tumors had a single aberration of *ErbB2*. Among 7 tumors with double aberrations, 5 had combinatorial copy number increases of *c-myc* and *ZNF217*, 1 had the combination *ErbB1/ErbB2*, and 1 had the combination *CCND1/ErbB2*. In all 3 tumors with triple aberrations, there was a combination of copy increases of *c-myc*, *ZNF217* and *ErbB2*. In 1 of the 2 tumors with 4 aberrations, the copy increases of the last three oncogenes were combined with a gain of *ErbB1*. In the second tumor affected by 4 aberrations, the combination was *c-myc/ZNF217/ErbB1/CCND1*.

Figure 1 represents the general pattern of sequential involvement of the analyzed genes in ovarian cancers; it exhibits the initial involvement of *c-myc*, followed by co-involvement of *ZNF217, ErbB2 (HER2), ErbB1* (*EGFR)* and *CCND1,* consecutively.

Correlation of the Copy Number Increases with the Pathological Parameters of the Tumors

We analyzed the incidence of the single and combinatorial copy number increases of the oncogenes in ovarian tumors in relation to different stages, grades and histology types. The results are represented in table 2 for stage, table 3 for grade, and table 4 for histology.

There were similar frequencies of the single aberrations in the three stages, but the incidence of combined aberrations increased with the advance of the stage without reaching significance (fig. 2A). The copy number increases of *c-myc* and *ZNF217* were present as single aberrations in all groups, whereas those of *ErbB1* were established only in stage I, and those of *CCND1* in stage II and III. The *ErbB2* copy number

Table 2. The incidence of single and combinatorial copy number increases of the oncogenes in tumors from different stages

	Aberrations					
Stage	None, n (%)	Single, n (%)	Double, n (%)	Triple, n (%)	Four, n (%)	Total, n
I	4 (30.8)	5 (38.4)	2 (15.4)	1 (7.7)	1 (7.7)	13
II	3 (42.8)	3 (42.8)	1 (14.4)	0	0	7
III	4 (22.2)	7 (38.9)	4 (22.2)	2 (11.1)	1 (5.6)	18

Table 3. The incidence of single and combinatorial copy number increases of the oncogenes in tumors of different grades

	Aberrations					
Grade	None, n (%)	Single, n (%)	Double, n (%)	Triple, n (%)	Four, n (%)	Total, n
Low	6 (37.5)	5 (31.2)	2 (12.5)	1 (6.2)	2 (12.5)	16
High	5 (22.7)	10 (45.5)	5 (22.7)	2 (9.1)	0	22

Table 4. The incidence of single and combinatorial copy number increases of the oncogenes in tumors of different histology

	Aberrations					
Histology	None, n (%)	Single, n (%)	Double, n (%)	Triple, n (%)	Four, n (%)	Total, n
Serous	7 (33.3)	5 (23.8)	6 (28.6)	2 (9.5)	1 (4.8)	21
Mixed	1 (20.0)	4 (80.0)	0	0	0	5
Non-serous	3 (25.0)	6 (50.0)	1 (8.3)	1 (8.3)	1 (8.3)	12

increase appeared as double aberration in 1 tumor from stage II and in 1 tumor from stage III. The remaining double aberrations comprised the combination *c-myc/ZNF217*. The latter was accompanied by an *ErbB2* gain in 1 tumor from stage I and in 2 tumors from stage III. An *ErbB2* gain was not present in stage I tumors affected by 4 aberrations.

The incidence of copy number changes of the five oncogenes – both single and combinatorial – was higher in high-grade tumors, except for the combination of 4 altered oncogenes (fig. 2B).

There were striking differences in the frequency of single aberrations between serous (23.8%) and other ovarian tumors: mixed (80.0%) and non-serous (50.0%). Double aberrations occurred 3 times more frequently in serous ovarian cancers (fig. 2C). Remarkably, all double aberrations in the serous group comprised *c-myc* and *ZNF217* copy number increases. The epidermal growth factor receptor *ErbB2* and *ERBB1* increases were combined in the single non-serous tumor affected by a double aberration. The unique non-serous tumors with 3 and 4 aberrations were from undifferentiated and clear cell histology, respectively.

Discussion

Genetic pathways defined at the chromosomal level are mirrored by genetic changes at the level of the gene. For example, p53 mutations are more common in cancer, accompanied by the loss of 17p13.1 [13]. The unstable genome is a hallmark feature of nearly all solid tumors. Cancer is known to be a disease of genomic instability with accelerated somatic evolution to a genomically heterogeneous population of cells naturally selected for their abilities to proliferate and invade while simultaneously evading host defenses. Genomic copy number increases are frequently found in cancers and are believed to contribute to their development and progression through amplification of oncogenes or more subtle gene dosage changes [3].

Here, we have analyzed the coexistence of copy number increases of five oncogenes – *CCND1, ErbB1, ErbB2, c-myc* and *ZNF217* – in 38 primary ovarian cancers of different histology, stages and grades. The five oncogenes were affected in 71.1% of ovarian cancers in single or combined aberrations. It is worth to consider their inclusion in a multi-panel for genetic diagnostics of ovarian cancer. Such testing panels exist for urinary bladder cancer [14], lung cancer [15], and prostate cancer [16]. The remaining 28.9% of the tumors in our set did not harbor alterations in the analyzed genes, but might have gains/amplifications in other oncogenes not included in the study. In spite of the limited number of samples analyzed in this study, the frequencies of gene alterations are consistent, to a great extent, with large-cohort studies like TMA analyses of up to 1000 tumors that have been done in our laboratory (data not shown).

We report the highest frequency of *c-myc* genetic gain/amplification, since it affected 42.1% of the ovarian tumors. Moreover, we observed sequential involvement of copy number alterations of the other genes in the presence of *c-myc* disruption. Recently, the common fragile site FRA8C was cloned and found to map close to the *c-myc* proto-oncogene [17]. According to the genomic database, there were at least two more fragile sites in the region, FRA8D (8q24.3) and FRA8E (8q24.1). It is well known that fragile sites exhibit signs of instability in tumor cells, and they have been implicated in cancer chromosome rearrangements causing deregulation of the genes in the regions [18]. Obviously, *c-myc* genomic imbalances, giving rise to the oncogene's dysfunction,

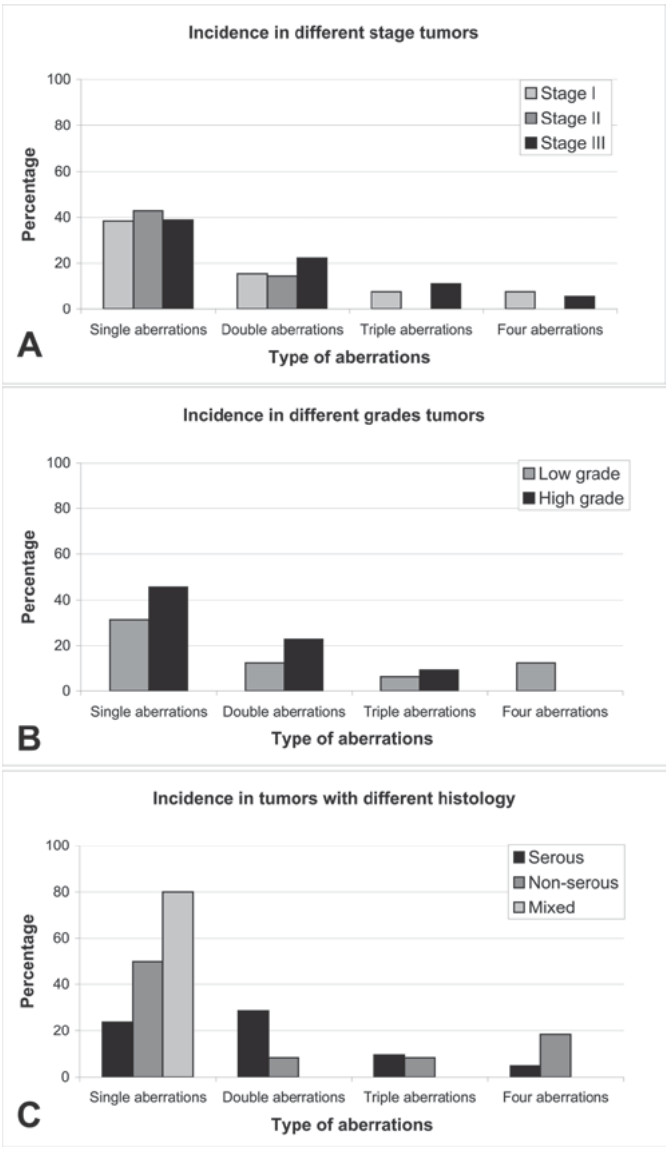

Fig. 2. The incidence of copy number increases of the analyzed genes in relation to clinico-pathological parameters: (**A**) tumor stage, (**B**) tumor grade, (**C**) histology.

emerged as an early event in ovarian carcinogenesis, due to the common instable site in the 8q24 locus. It was followed by copy number changes in the other potential oncogenes analyzed in our study – *ZNF217*, *ErbB2, ErbB1* and *CCND1*. A similar pattern of sequence was discovered in breast cancer progression, where *c-myc* amplification was an early event while *ZNF217* and *ErbB2/neu* amplifications were suggested to play a role in the later stage of tumor development [19]. The copy numbers of *ErbB2, ErbB1* and *ZNF217* were altered more often together with other genes in our study, a fact supporting the idea of their later involvement in the process of ovarian carcinogenesis. There are reports of combined aberrations of HER receptor genes and c-Myc, which is their potential effector [20, 21], and even combined anti-gene therapy has been elaborated against *ErbB2* and *c-myc* [22]. Another frequent combined alteration was found between *erbB-1* and *erbB-2*, being used as an independent prognostic factor for specific survival [23, 24]. According to others, there are no combined alterations of *erbB-1* and *erbB-2* [25], or the changes of *erbB-1* are commonly accompanied by those of *erbB-3* [26]. Ambiguous data were reported for *CCND1*; its amplification is correlated to 20q13.2 amplification according to some authors [27] or the two genes were affected independently according to others [28, 29].

We were interested in verifying whether some of the anomalies we had analyzed were diagnostic in terms of separating tumors of different stage, grade or histological types. We discovered that *c-myc* and *ZNF217* were altered in all stages, whereas *ErbB2* was altered mainly in advanced stages. Moreover, *ErbB2* copy number increases were not detected as single aberrations in ovarian tumors in our study. The combination between amplification of *c-myc* and *ErbB2* was found to be associated with poor patient survival and was suggested to be a worse prognostic indicator of ovarian cancer development in another study [30].

High-grade ovarian tumors were associated with a higher incidence of copy number alterations in our study. Assessing the DNA copy number changes in 33 high-grade serous carcinomas and 10 low-grade serous tumors by using a genome-wide technique, a single-nucleotide polymorphism array, Nakayama et al. found that high-grade serous carcinomas showed widespread DNA copy number changes in comparison to low-grade tumors [31]. It is evident that the poorly differentiated tumors are genetically more instable.

Combined copy number increases of *c-myc* and *ZNF217* (20q13) were consistently observed in ovarian serous cancers in our study and were not detected in the non-serous histological type. This coexistence does not seem to be occasional, but a fact that indicates a strong correlation between mutations of the two genes. Yang and colleagues suggested that the key gene of the 20q13.2 amplicon *STK15* activates human telomerase reverse transcriptase (hTERT) by targeting the *c-myc* transcription factor [32]. We suggest that the copy numbers of the two genes are very often increased together, especially in serous ovarian cancers; probably, this is one of the possible mechanisms for dysregulation of this pathway.

In conclusion, our results revealed a combination between copy number increases of *c-myc* and *ZNF217*, associated with serous histology. Genomic imbalances of *c-myc* are early events in ovarian tumorigenesis, and those of *ErbB2, ErbB1* and *ZNF217* (20q13.2) are late events. The data from this combined analysis of the five oncogenes could be used as a basis for considering a combined approach in molecular-based therapy of ovarian cancer.

Conflict of Interest

The authors did not provide a conflict of interest statement.

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